Affinity for measles virus anti-haemolysin of a residual immunoglobulin M in sera of some patients with multiple sclerosis

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SUMMARY

HEp$_2$ cells persistently infected with measles virus were treated with trypsin to remove haemag-glutinin (HA) and examined unfixed or fixed in acetone by fluorescent antibody methods, comparing those sera specific for structural antigens of the virus. Staining patterns combined with the blocking of specific immunofluorescence indicated that IgG specific for measles virus haemolysin could be recognized in multiple sclerosis (MS) sera and that in some sera from which rheumatoid factor had been removed, a residual IgM (MS-IgM) was absorbed to measles virus-infected cells and showed the same specificity in blocking tests as measles virus anti-haemolysin. MS-IgM could be removed from sera by absorption with latex particles coated with human IgG and would seem to be anti-globulin with preferential affinity for anti-haemolysin.

INTRODUCTION

It is well-known that sera from patients with multiple sclerosis (MS) have, on average, slightly higher titres of antibody against measles virus than matched sera from patients with other neurological diseases and from healthy people (Adams & Imagawa, 1962; Sever *et al.*, 1971; Salmi *et al.*, 1973). The reason for this abnormal increase, which is two- to three-fold at most when large numbers of observations are surveyed (Fraser, 1975), is not known, but there is no substantiated record of measles virus persisting in MS patients which could account for it. Similar differences in antibody titre to other viruses as well as measles virus are occasionally found in sera from MS patients, but more often in sera from patients with autoimmune disease (Phillips & Christian, 1969) and also with chronic disease of the liver (Triger *et al.*, 1972). It is possible that one fundamental abnormality of the immune response could account for the serological findings in the three different kinds of disease. The exact specificity of any abnormal measles antibody present in multiple sclerosis is therefore worth determining as a first step towards comparing these abnormal responses.

It has been shown by the fluorescent antibody method that in MS sera, from which rheumatoid factor has been removed by heat-aggregated gamma-globulin, there is an IgM which is specific for the surface of unfixed measles virus-infected cells (Haire, Fraser & Millar, 1973). The existence of this IgM has been confirmed in the sera of MS patients by a different method (Karaseva *et al.*, 1974). We have described this as a virus specific anti-haemolysin (Fraser, 1977). The principal characteristics of MS-IgM are as follows: it is present in upwards of 40% of MS patients (Haire, 1977); it persists in sera which have been absorbed with sufficient heat-aggregated gammaglobulin to remove rheumatoid factor (Haire *et al.*, 1973); its titre is low and may fluctuate from time to time, so that it may be absent from one or other sample of serum from the one patient; the fluctuations are not related to the state

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of the disease (Haire, 1977). Pretreatment of cells with phospholipase C apparently removed the receptors for MS-IgM, but not the receptors for the measles-specific IgM found in sera of patients in the early stages of measles. Pre-treatment with trypsin, which destroys measles virus haemagglutinin (HA) (Norrby, 1962; Periés & Chany, 1962) did not abolish antigens that can be stained with MS-IgM (Fraser, Shirodaria & Haire, 1974).

In this paper we demonstrate that adsorption of MS-IgM to infected cells is not related to measles virus HA, but does depend on the antigen of haemolysin present on the surface of HEp$_2$ cells persistently infected with measles virus. The structural antigens at the cell surface were identified by means of fluorescent antibody staining with monospecific antisera (Fraser *et al.*, 1978) and the specificity of MS-IgM was determined by blocking of specific MS-IgM fluorescence by one of these sera.

MATERIALS AND METHODS

Carrier cells. The 34 line of HEp2 cells chronically infected with measles virus (Gould & Linton, 1975) was propagated every 3 days in Eagle's medium with 10% foetal calf serum and used at passes 248–278. It carried surface antigens, detectable by immunofluorescence on 25–35% of cells, and all the experiments reported here were carried out on cultures which carried nucleocapsid antigen in about 99% of cells.

For immunofluorescence tests on the 34 line, 2×10^5 cells, removed from culture bottles by a solution of 0·05% trypsin and 0·02% versene, were plated in growth medium above circular glass coverslips in 50 mm petri dishes. They were incubated for 48 hr at 36°C and examined either unfixed or after being fixed in acetone for 6–8 min at room temperature.

Preliminary tests showed that suspensions of 34 cells removed from culture bottles by 0·02% versene could be used to demonstrate measles virus antigens at the cell surface. Cells were treated with 0·004% crystalline trypsin for 40 min to remove haemagglutinin from the surface of all but a fraction of 1% of cells and then fixed with 4% para-formaldehyde which stopped trypsin activity (Ehrnst & Sundqvist, 1975). To achieve accurate timing and uniform activity of the enzyme on suspended cells, the use of trypsin–versene solution to suspend monolayered cells was avoided.

Lytic infection. This was studied on HEp$_2$ cells grown in the same medium as line 34 plus tryptose phosphate broth, infected with Edmonston-derived measles virus (Gould, 1974) and maintained in medium with 1% calf serum for 36 hr at 36°C.

Sera. Four guinea-pig antisera, specific for either haemagglutinin (HA), haemolysin (HL), nucleocapsid (NC) or membrane protein (MP) of measles virus were used in the immunofluorescence tests. The sera did not cross-react in anti-haemagglutinin, anti-haemolysin or complement-fixation tests, and each showed a characteristic and distinct pattern of fluorescent-antibody staining of measles virus-infected HEp$_2$ cells when tested by the indirect fluorescent antibody method (Table 1). Human sera were inactivated at 56°C for 30 min and absorbed with aggregated human IgG so as to be free of rheumatoid factor when tested by immunofluorescence of acetone-fixed virus-infected cells (Shirodaria, Fraser & Stanford, 1973).

Fluorescent antibody staining. Direct and indirect methods were used. For direct staining FITC conjugates of guinea-pig sera were prepared by standard methods, absorbed two or more times with human liver powder and cultured HEp$_2$ cells, and used at a dilution which was free of non-specific staining. Specificity was controlled by noting the absence of staining on non-infected cells, or on cells infected with other viruses and by the removal of the specific staining effect after conjugated sera had been absorbed with measles virus-infected tissues. Indirect staining was carried out with absorbed FITC-conjugated antisera to guinea-pig Ig or human IgG and IgM (Nordic Immunological Laboratories, Maidenhead, Berks. and Wellcome Reagents Limited, Beckenham, Kent) which had been re-tested for specificity before use (Chantler & Haire, 1972), neither of which cross-reacted with the other globulins at the dilutions used. This made it possible to perform reciprocal blocking tests between guinea-pig and human sera. About 15 to 20 staining units of each serum (twenty times the concentration of the end-point) were used to stain cells for counting. Specific anti-nucleocapsid serum was occasionally added to anti-HA or anti-HL sera in order to identify infected cells by the characteristic inclusion bodies.

In reading our results the following points should be borne in mind: two structural antigens of measles virus are present at the infected cell surface, HA and HL; of these, HA is resistant and HL is sensitive to acetone; and fluorescent antibody staining of acetone-fixed tissue cannot be produced by human anti-HL serum.

Two structural antigens, NC and MP, are situated intra-cellularly or in the cell membrane and cannot normally be stained by antibody at the surface of unfixed infected cells. Adult human sera usually contain more anti-HL than anti-HA and absorption of human serum with acetone-fixed measles virus-infected cells removes antibodies to all structural antigens except anti-HL, which remains with titre undiminished.

Guinea-pig anti-HL does not contain two antibodies, but it reacts with antigen which is acetone-sensitive in some cells and acetone-resistant in others (Fraser *et al.*, 1978). Some of the features re-appear in the data presented here and the staining patterns of the sera are given in Table 1.

Globulin-coated latex particles, a commercial preparation (Hyland RA test, Batch 0376 No. 42AA, 1977), was washed twice before being used as an absorbant for human sera.

TABLE 1. Properties of measles virus-specific sera used in fluorescent antibody staining

Serum	Abbreviation	Staining property on infected cells	Staining of uninfected cells
Guinea-pig anti-haemagglutinin	Anti-HA	Stains HA only at surface of unfixed cells and at surface and in cytoplasm of fixed cells	None
Guinea-pig anti-haemolysin	Anti-HL	Stains acetone-sensitive HL at cell surface of unfixed cells and an acetone-resistant component, not HA, in some acetone-fixed cells	None
Guinea-pig anti-nucleocapsid	Anti-NC	Stains nothing on unfixed cells, but inclusion bodies in all fixed cells and NC at margin of productively infected cells only	None
Guinea-pig anti-membrane protein	Anti-MP	Stains margin of fixed infected cells brightly. Does not usually stain unfixed cells	None
Human serum unabsorbed	—	Stains HA and HL at surface of unfixed cells. Stains all antigens in fixed cells	None
Human serum absorbed with acetone-fixed measles virus-infected cells	—	Stains acetone-sensitive HL at surface of unfixed cells. Does not stain fixed cells, unlike guinea-pig anti-HL	None

RESULTS

Acetone-sensitivity of virus antigens

In view of the sensitivity to acetone of MS-IgM staining of measles virus-infected cells and of measles virus haemolysin (see the Materials and Methods section), a comparison was made between the acetone sensitivity of surface antigen on lytically infected monolayers and on persistently infected HEp_2 cells in suspension. Growth conditions, fixation and staining were the same for the two cultures.

A guinea-pig serum containing 15 staining units of anti-HL but no anti-HA was used to detect HL antigen. A similar concentration of anti-HA was used as a control. Simultaneous or separate application of guinea-pig anti-NC to mark infected cells (see the Materials and Methods section) was essential in the lytic infection, where 60% or more of cells might not be infected.

HA antigen at the cell surface was resistant, and HL antigen sensitive, to acetone fixation in both lytic and persistent infection (Table 2). The number of cells showing surface staining after acetone fixation was always greatly reduced and cell counts were alike when any one serum was used in different experiments. Variation occurred between sera and when anti-nucleocapsid serum was added as a marker, for some productively infected cells show marginal staining of NC which is confusing in fixed cultures and raises the number of apparently acetone-resistant cells. The lytic and persistently infected cultures showed a marked difference in the proportion of infected cells which showed acetone-sensitivity of the HL antigen at the cell surface; 52–64% in the lytic culture and 85–91% in the persistent infection.

Trypsin-sensitivity

The binding of MS-IgM to the surface of measles virus-infected cells on glass is resistant to a moderate degree of pre-treatment of cells with trypsin (Fraser *et al.*, 1974). Cells in the lytic cycle could not be tested in suspension because they always lysed before treatment with trysin of the surface antigens was completed. In persistently infected cells, the HA antigen was completely removed from the cell surface by trypsin, whilst the HL antigen remained or was slightly enhanced in sensitivity to specific antiserum (Table 3).

TABLE 2. Surface immunofluorescence of measles virus-infected HEp$_2$ cells

Percentage of infected cells* showing fluorescence (500–900 cells counted) when treated with anti-haemagglutinin (anti-HA) or anti-haemolysis (anti-HL)

	Lytic infection				Persistent infection (carrier culture)			
	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	Anti-HL	Anti-HA	Anti-HL	Anti-HA	Anti-HL	Anti-HA	Anti-HL	Anti-HA
Unfixed	20·8	18·1	20·7	19·0	26·2	22·3	35·6	34·5
Acetone-fixed	7·5	18·3	9·8	20·3	1·3	24·2	5·2	32·2
Percentage acetone-sensitive	64·0	0	52·0	0	91·0	0	85·0	6·0

* Infected cells recognized by staining of intra-cytoplasmic aggregates with anti-NC.

TABLE 3. Effect of trypsin on surface immunofluorescence of carrier culture of measles virus

	Percentage of cells giving fluorescence at membrane by (400 counted)*	
	Anti-HL	Anti-HA
Untreated cells not fixed	18·5 (8–32)*	17·6 (12–24)
Trypsin-treated fixed cells†	25·0 (13–45)*	1·0 (< 1%–1%)

* Mean (and extremes) of thirteen experiments.
† Fixation alone does not alter the proportion of cells stained by anti-HA.

MS-IgM staining of persistently infected cells

The titre of MS-IgM ranges from 1:4 to 1:64 (Haire, 1977). Consequently, the number of persistently infected cells which can be specifically stained by MS-IgM is only about one fifth of the 26–35% stained by high titre antisera to measles virus (Table 2). In six sera tested, the mean number of positive cells was 4·5 before, and 4·4 after, trypsin treatment, showing that the receptor for MS-IgM is not HA but one which is like HL.

MS-IgM and IgG of corresponding specificity

When human serum is applied to untreated measles virus-infected cells, it is not possible to determine from the pattern of surface fluorescence which virus antigens are being stained. On trypsin-treated, persistently infected cells, surface staining by anti-HA can be excluded, and when this was done, an IgG which had the same serological reactivity as MS-IgM was demonstrated. This was called MS-IgG for convenience. It was present in sera from twenty MS patients, mean titre 185·5, mean log titre 2·682 log s.d. 0·3449, and from twenty normal subjects matched for age and sex, mean titre 65·9, mean log titre 1·8191 log s.d. 0·4322, and the titre was significantly higher in MS patients, $P < 0·001$ by paired t-test. There was a difference in titre between ten sera containing MS-IgM, mean titre 179·1, mean log titre 2·2531 log s.d. 0·2221, and ten sera from MS patients which did not have detectable MS-IgM, mean titre 253·3, mean log titre 2·4037 log s.d. 0·3236, but it was not statistically significant, $P > 0·1$ by Student's t-test.

The behaviour of MS-IgG in five sera from MS patients was compared with the reaction of mono-pecific antibodies to HA and HL on trypsin-treated, acetone-treated and non-treated carrier cells.

The proportions of fluorescent cells given by the anti-HL serum and by the five MS sera before and after each treatment were similar (Table 4), but trypsin treatment removed all HA from the infected cell surface (column 1). Trypsin treatment or acetone fixation each reduced the number of surface fluorescing cells given by MS sera and by anti-HL serum by about one half, but treatment with both reagents abolished all receptor activity. Thus, about one half of the cells that could be stained in the carrier line had a specific receptor for anti-HL which was trypsin-resistant and acetone-sensitive. The five human sera clearly behaved like anti-HL and not like anti-HA.

Fluorescence blocking tests

Blocking of immunofluorescence was carried out on unfixed lytically infected HEp_2 cells by the indirect fluorescent antibody method between six MS sera and guinea-pig sera, and by the direct method between guinea-pig sera. Blocking by MS-IgG was tested against four of the six MS-IgM sera, using two MS sera not containing MS-IgM and from which all anti-HA had been removed by absorption methods. Blocking of all six MS sera gave consistent results. MS-IgM was not blocked by anti-HA, anti-NC or anti-MP sera, but was blocked by anti-haemolysin. Four of the MS-IgM sera were blocked by MS-IgG a result which requires discussion. Reciprocal blocking by heterologous mono-specific guinea-pig sera was absent; autologous blocking was very distinct.

TABLE 4. Surface fluorescence of measles virus carrier cells to anti-HL and MS-IgG by indirect immunofluorescence

Treatment of carrier cells	Percentage fluorescent cells given by:						
	Anti-HA	Anti-HL	MS1	MS2	MS3	MS4	MS5
None	27·0	26·5	21·5	19·0	12·0	18·0	17·0
Acetone	27·0	16·0	10·0	8·0	5·5	7·0	6·0
Trypsin	< 1·0	7·5	11·0	9·0	11·0	9·0	9·0
Trypsin, then acetone	< 1·0	0	0	1·0	0	0	0

Absorption of MS-IgM by human IgG on latex particles

It has been reported recently by Salmi *et al.* (1978) that virus-specific IgM could not be detected in the sera of multiple sclerosis patients by radioimmunoassay, provided that measles virus-specific IgG had been removed by adsorption to staphylococcal A protein. To determine the nature of our MS-IgM further, we subjected seven sera with MS-IgM titres, range 6–64 and depleted of rheumatoid factor, shown by testing on acetone-fixed virus-infected cells (Shirodaria *et al.*, 1973) to absorption by latex particles coated with human IgG. A concentration of 2% latex (v/v) and absorption temperatures of 4°C, 16°C and 37°C were applied for 30 min. Fluorescent staining by IgM was completely removed from five sera, titres 6–40, and almost completely removed from two sera, titres 48 and 60, indicating that MS-IgM was anti-globulin. Sensitivity of haemolysin to acetone fixation probably explains the fact that none of our seven sera gave positive staining for MS-IgM on fixed tissue.

DISCUSSION

There are two main conclusions from our results. The first is that IgM in MS sera is demonstrably an anti-globulin that is removed from sera by absorption with human IgG on latex particles, and not a measles virus-specific antibody as we had suggested. This is in accord with the observation of Salmi *et al.* (1978) that no IgM is found to unite with measles virus antigen if virus-specific IgG is first removed. Our method of assaying rheumatoid factor on acetone-fixed measles virus-infected cells was inadequate for detecting all IgM anti-globulin, and whether MS-IgM is a residual rheumatoid factor of low avidity

or an entirely different IgM remains to be investigated. Haemagglutinin-specific and haemolysin-specific IgG molecules may have different capacities to absorb MS-IgM for they are known to activate complement in different ways (Ehrnst, 1977). It would be interesting to know if they belong to different sub-classes of IgG, as studies in CSF suggest (Palmer, Minard & Cawley, 1976; Vandvik, Natvig & Norrby, 1977).

The second conclusion which seems to be reasonably well established is that MS-IgM has a preferential affinity for measles virus anti-haemolysin. Considering it as an anti-globulin, we can state that it does not bind to anti-haemagglutinin because staining is abolished by acetone treatment which leaves the union of haemagglutinin and its antibody unaffected; as a rule it does not produce fluorescent IgM staining of nucleocapsid inclusions and nucleocapsid does not appear at the surface of the unfixed infected cells, where MS-IgM staining is characteristic (Fraser *et al.*, 1978), so it is not specific for anti-nucleocapsid; the antigen of membrane protein is acetone-resistant in the fluorescent antibody test, so abolition of MS-IgM fluorescence by acetone fixation of tissue would seem to exclude the union of MS-IgM and anti-membrane protein. The fact that MS-IgM is readily removed from serum by IgG on latex particles makes it unlikely that it is specific for the idiotype of anti-measles virus haemolysin, which is known to exist quite separately from anti-haemagglutinin (Norrby & Gollmar, 1972; 1975).

The conclusion that MS-IgM unites with anti-haemolysin is fully supported by fluorescence blocking, which shows specific inhibition of MS-IgM fluorescence by guinea-pig antisera to haemolysin only, and not by antisera to haemagglutinin, nucleocapsid or membrane protein.

Blocking by MS-IgG, which is itself specific for measles virus haemolysin, is a paradoxical result considering that we now know MS-IgM to be an anti-globulin. Shirodaria (unpublished observations) has found quite regularly that zoning or inhibition of fluorescence occurs at the lowest dilutions of high titre rheumatoid sera when titrating rheumatoid factor by immunofluorescence on virus-infected cells. It may be that something of the sort is occurring here. It could be that an IgG anti-globulin exists which would block RF-like IgM, but we are not in a position to test this possibility.

The nature of the acetone-resistant component of haemolysin revealed by guinea-pig anti-haemolysin (Tables 1 and 3) and human sera (Table 4) need not concern present conclusions. Previous studies have shown that the acetone-sensitive determinant is haemolysin. Anti-HL has been noted by others (Salmi, Norrby & Panelius, 1972) as one of the best indicators of the difference between MS and non-MS sera. How this disproportionate increase of the haemolysin-specific IgG in MS sera, which we have confirmed by the fluorescent antibody technique, comes about is not yet known. The only alternative explanation to the persistent presence of measles virus or a related antigen would seem to be an alteration of immunological control of the normal persisting measles virus antibody response by some unrecognized factor in MS and in autoimmune diseases, for example, a loss or inhibition of specific suppressor cells.

The recognition of MS-IgM as anti-globulin also brings to mind the unknown aetiology of rheumatic diseases, in which increased titres of virus antibody have been recorded. Comparison of the immunological changes in MS with those of rheumatoid arthritis might be rewarding.

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